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## **Rapid *RHD* zygosity determination using digital PCR**

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**Keywords:** Zygosity, digital PCR (dPCR), long-range PCR (LR-PCR), next generation sequencing (NGS), genotype, serology and single nucleotide polymorphisms (SNPs).

**List of Abbreviations:** Hemolytic disease of the newborn and fetus (HDFN), real-time PCR (qPCR), digital PCR (dPCR), minutes (mins), double-stranded DNA (dsDNA), droplet digital PCR (ddPCR), *RHD* exon 5 (*RHD5*), *RHD* exon 7 (*RHD7*), High Performance Liquid Chromatography (HPLC), seconds (s), long-range PCR (LR-PCR), next generation sequencing (NGS), Ion sphere particles (ISPs), Ion Personal Genome Machine™ (Ion PGM™), Variant Caller Files (VCF), human

24 genome 19 (hg19), Integrative Genomics Viewer (IGV), the Single Nucleotide  
25 Polymorphism Database (dbSNP), single nucleotide polymorphism (SNP), Browser  
26 Extensible Data (BED), cell-free DNA (cfDNA), National Center for Biotechnology  
27 (NCBI).

28 **Human Genes:** Reference genes: *AGO1* (argonaute RISC catalytic component 1,  
29 HGNC: 3262) (or *AGO1* (eukaryotic translation initiation factor 2C, 1)). Target genes:  
30 *RHD* (Rh blood group, D antigen, HGNC: 10009) (or Rhesus blood group, D  
31 antigen).

## Abstract

**Background:** Paternal zygosity testing is used to determine the hemi- or homozygosity of *RHD* in pregnancies at risk of hemolytic disease of the newborn and fetus (HDFN). Currently, this is achieved using real-time PCR or the RH box PCR, which can be difficult to interpret and unreliable, particularly for black African populations.

**Method:** DNA samples extracted from 58 blood donors were analysed using two multiplex reactions for *RHD* specific targets against a reference (*AGO1*) to determine gene dosage using digital PCR. Results were compared to serological data and the correct genotype for two discordant results was determined by long range-PCR, next generation sequencing and conventional Sanger sequencing.

**Results:** The results show clear and reliable determination of *RHD* zygosity using digital PCR and revealed that four samples did not match the serologically predicted genotype. Sanger sequencing and long range-PCR followed by next generation sequencing revealed that the correct genotypes for samples 729M and 351D, which were serologically typed as  $R_1R_2$  (DCE/dCE), were  $R_2r'$  (DcE/dCE) for 729M and  $R_1r''$  (DCE/dcE),  $R_0r^y$  (Dce/dCE) or  $R_zr$  (DCE/dce) for 351D, in concordance with the digital PCR data.

**Conclusion:** Digital PCR provides a highly accurate method to rapidly define blood group zygosity, and has clinical application in the analysis of Rh phenotyped or genotyped samples. The vast majority of current blood group genotyping platforms are not designed to define zygosity, and thus this technique maybe used to define paternal *RH* zygosity in pregnancies at risk of HDFN, and distinguish between homo- and hemizygous *RHD* positive individuals.

## Introduction

Of the 36 blood group systems Rh is the most complex at the genetic level and is the major cause of hemolytic disease of the fetus and newborn (HDFN) and a major cause of transfusion reactions during alloimmunization events. The *RH* genes, *RHD* and *RHCE*, are well characterized at the genetic level, with a combination of SNPs in *RHCE* being responsible for the C/c and E/e polymorphisms, whilst gene deletion and hybrid *RHD-RHCE* genes are responsible for D-negative phenotypes, plus SNPs and hybrid genes being responsible for partial and weak D phenotypes (1, 2). All known mutations have been well catalogued and best described in the RhesusBase resource (3).

Paternal *RHD* zygosity testing is important for prenatal management of alloimmunized women. Where fathers are homozygous D- there is no risk of HDFN for the current pregnancy or subsequent pregnancies that may follow. Pregnancies to homozygous D+ fathers (with the assumption of paternity) will by definition carry RhD positive fetuses, and can be considered for more focused clinical management. For hemizygous D+ fathers non-invasive prenatal testing (NIPT) is required for a definitive diagnosis.

Previously published methods for *RHD* zygosity testing have included real-time PCR (qPCR) assessment of *RHD* gene dosage, assessment of the hybrid *Rhesus box* found in D-negative individuals with the *RHD* gene deletion genotype and allele-specific PCR methods, as well as mass spectrometry-based methods. (2, 4-10). Zygosity testing targeting the hybrid *Rhesus box* found in *RHD*-deletion type cde haplotypes is complicated because of differences in the hybrid box amongst individuals of African descent (5, 11).

The incidence of common *RH* haplotypes in Caucasian, African black and Asian populations has been defined serologically. In *RHD* positive individuals the D<sub>Ce</sub> haplotype is prevalent in Asian (73%) and Caucasian (42%) populations, but in African black populations the D<sub>Ce</sub> haplotype has a higher incidence (59%) (12). *RHD* negative individuals are rarely found in Asian populations (<4%), but the d<sub>Ce</sub> haplotype is frequently found in Caucasian (39%) and African black (20%) populations (12). Rare haplotypes such D<sub>C</sub>E, d<sub>C</sub>e, d<sub>C</sub>E and D<sub>C</sub>e are considerably less prevalent with frequencies of 0.24%, 0.98%, 1.19% and 2.57% respectively, in Caucasian populations (12). However, it has been difficult to define the precise population frequencies of the various *RH* haplotypes due to the inability to differentiate between hemi- or homozygous individuals. For example, an individual with the phenotype D<sub>Ce</sub> would be designated as the most common presumed genotype D<sub>Ce</sub>/D<sub>Ce</sub> rather than D<sub>Ce</sub>/d<sub>Ce</sub>. Thus presumed genotype, based on probability, is the manner in which donor and patient red cells are labelled. Zygosity determination of the above would define which presumed genotype (D<sub>Ce</sub>/D<sub>Ce</sub> or D<sub>Ce</sub>/d<sub>Ce</sub>) (two copies of the *RHD* gene versus one copy of the *RHD* gene) is carried by a particular individual.

Previously we have applied digital PCR (dPCR) to the analysis of free fetal DNA derived from maternal plasma (13). In this study we have utilized dPCR as a more accurate quantitative PCR method than conventional qPCR to define *RHD* zygosity. We found rare haplotypes in a relatively small cohort of samples and identified that for three samples (plus one weak D sample) their predefined and labelled presumed genotype was indeed incorrect.

## **Materials and Methods**

## **Study Participants**

Human whole blood samples ( $n = 79$ ) were supplied by the National Health Service Blood and Transplant (NHSBT) (Bristol, UK) (donated with informed consent) and transported to NHS Plymouth Hospitals Trust, Plymouth, UK for collection.

## **Sample Processing**

Samples were processed in two ways. Human whole blood samples ( $n = 25$ ) were collected in EDTA tubes (5-10 mL total blood volume) and centrifuged at 1 600xg for 10 minutes (min) at room temperature. The plasma was carefully removed and transferred to a 15 mL tube. The plasma was then re-centrifuged at 16 000xg for 10 min. All samples were processed within 48 to 96 hours of collection and plasma aliquots (1 mL) were stored at  $-80^{\circ}\text{C}$ .

Human whole blood samples ( $n = 54$ ) were collected in EDTA tubes (5-10 mL total blood volume) and centrifuged at 2 500xg for 10 min at room temperature. The buffy coat layer was carefully removed and transferred to a 1.5 mL tube for immediate processing to genomic DNA (gDNA). All blood samples were processed within 48 to 96 hours of blood collection.

## **DNA extraction from plasma**

Plasma extractions were performed as non-pregnant controls from maternal plasma experiments (13) and were further utilised in this study. DNA was extracted from two 1 mL aliquots of plasma using the QIAamp Circulating Nucleic Acid (CNA) kit (Qiagen, West Sussex, UK) using the QIAvac 24 Plus (Qiagen). The extraction process was as the manufacturer's protocol and each sample was eluted in 60  $\mu\text{L}$  Buffer AVE (RNase free water containing 0.04% (w/v) sodium azide). No DNase or

127 RNase treatment was used. Following DNA extraction, samples were quantified on  
128 the Qubit® 2.0 Fluorometer (Life Technologies, Paisley, UK) using the Qubit®  
129 dsDNA HS assay kit (Life Technologies). Samples were stored at -20°C as 60 µL  
130 aliquots for up to four weeks.

#### 131 **DNA extraction from buffy coat**

132 For *RHD* intronic SNP sequencing, gDNA was extracted from buffy coats using the  
133 QIAamp DNA Blood Mini kit (Qiagen) according to the manufacturer's instructions.  
134 DNA was eluted in 200 µL Buffer AE and incubated at room temperature for 5 min  
135 before centrifugation at 11 865xg for 1 min. For the *RHD* long-range PCR (LR-  
136 PCR), gDNA was extracted from buffy coats using the Gentra® Puregene® Blood kit  
137 (Qiagen) according to the manufacturer's instructions for RNA-free DNA. As the  
138 buffy coat contained red blood cells, RBC Lysis Solution was used. Each sample  
139 was eluted by adding 300 µL of DNA hydration solution and mixed vigorously for 5  
140 seconds, followed by incubation at 65°C for 1 hour. The tube was then incubated at  
141 room temperature overnight with gentle shaking in order to mix the gDNA with the  
142 DNA hydration solution. Finally, the pure gDNA was transferred into a new 1.5 mL  
143 tube and stored at -20°C. Following DNA extraction, samples were quantified on the  
144 Qubit® 2.0 Fluorometer (Life Technologies) using the Qubit® double-stranded DNA  
145 (dsDNA) High Sensitivity assay kit (Life Technologies).

#### 146 **PCR Primers and Probes for dPCR**

147 Two multiplex reactions were tested on the QX100™ droplet digital PCR (ddPCR)  
148 platform (Bio-Rad Laboratories, Herfordshire, UK) for *RH* zygosity testing (Table 1),  
149 as previously described in Sillence *et al.* (13). The oligonucleotide sequences (High  
150 Performance Liquid Chromatography (HPLC) purified, Eurofins Genomics, Germany)



and amplicon sizes for all target (FAM-labelled) and reference (HEX-labelled) regions are shown in Table 1. Prior to zygosity testing, primer annealing temperatures (56°C to 60°C) were optimised for both multiplex reactions (see Supplemental Figure 1). The results in Supplemental Figure 1a showed successful droplet separation of the *RHD5* (FAM) target at all annealing temperatures, but the *AGO1* (HEX) reference showed sub-optimal separation at 60°C. Droplet separation for the *RHD7* (FAM)/ *AGO1* (HEX) multiplex reaction (see Supplemental Figure 1b) demonstrated the same pattern as previously discussed for the *RHD5* (FAM)/*AGO1* (HEX) multiplex reaction. However, the optimal ratio was visible at 58.4°C (0.995). Therefore 58°C was determined to be the optimum annealing temperature for both multiplex reactions.

## **dPCR**

The dPCR reactions were conducted in duplicate and run on the QX100™ Droplet Generator (Bio-Rad) following manufacturer's instructions (see Sillence *et al.* (13)). Plasma extracted samples were not diluted and a standard volume of template DNA (5 µL) was added. Samples extracted from buffy coat were diluted and 50ng of DNA was added to each 20 µL reaction and a non-template control (NTC) was included in each assay.

## **Data Analysis for dPCR**

The raw fluorescent data from the ddPCR platform was analysed using the Bio-Rad QuantaSoft v1.2 software. Once thresholds for each sample had been set manually using the 1D amplification plot, positive and negative droplets were determined (see Supplemental Figure 1). The concentration was then determined by the software using Poisson statistics (95% confidence interval) for each sample. The ratio of the

175 target (*RHD5*-FAM and *RHD7*-FAM) over the reference (*AGO1*-HEX) for each  
176 sample was calculated as follows: FAM (copies/  $\mu$ L)/HEX (copies/  $\mu$ L). All statistical  
177 analysis was performed using Mann Whitney U Test (SigmaPlot Version 12.5) and  
178 significance was accepted at  $p < 0.05$ .

#### 179 ***RHD* LR-PCR and Next generation sequencing (NGS)**

180 gDNA samples from blood donors of different phenotypes were tested using LR-PCR.  
181 Three PCR products were designed to cover the entire *RHD* gene (Table 2). The  
182 HPLC-purified primers were from Eurofins MWG Operon (London, United Kingdom).  
183 The PCR reaction contained a final 1x concentration of PrimeSTAR GXL Buffer  
184 (Takara, Japan), 200  $\mu$ M dNTP mixture, 0.2  $\mu$ M of each primer and 1.25 unit  
185 PrimeSTAR GXL Polymerase per 50  $\mu$ L and 500ng DNA per reaction. A two-step  
186 protocol was performed as 25 cycles of 98°C for 10 s and 68°C for 24 min, final hold  
187 at 4°C. The amplicons were purified on 0.5% w/v agarose gel in 1X TAE buffer. The  
188 long amplicons were purified by Agencourt® AMPure® XP beads (Beckman Coulter,  
189 High Wycombe, UK) to ensure removal of primer dimers, polymerase and free  
190 nucleotides. The samples were eluted in 50  $\mu$ L nuclease-free water. Purified  
191 amplicons were quantified by Qubit® dsDNA Broad-Range assay kit (Life  
192 Technologies) to allow the starting concentration of the sequencing libraries to be  
193 100 ng. Following quantification, enzymatic fragmentation was completed using the  
194 Ion Xpress™ Plus Fragment Library Kit (Life Technologies) resulting in fragments of  
195 ~200bp. Next, the fragments were ligated with barcoded adapters, which add about  
196 70bp to the fragments. P1 and Ion Xpress™ Barcode X adapters from the Ion  
197 Xpress™ Barcode Adapters Kit (Life Technologies) were used to distinguish the  
198 samples when pooled prior to sequencing. The adapter-ligated library was size  
199 selected by SPRIselect® reagent kit (Beckman Coulter, High Wycombe, UK). After

200 each step (fragmentation, ligation and size selection), purification was conducted  
201 using magnetic beads and the integrity, size distribution, concentration and quality of  
202 the library in those steps was checked using the Agilent® 2100 Bioanalyzer®  
203 instrument and Agilent High Sensitivity DNA Kit (Agilent Technologies UK Limited,  
204 Stockport, UK).

205 Template-positive ion sphere particles (ISPs) containing clonally amplified DNA were  
206 prepared by the Ion Personal Genome Machine™ (PGM™) Template OT2 200 Kit  
207 (for 200 base-read libraries) (Life Technologies) with the Ion OneTouch™ 2 System.  
208 Then the percentage of template-positive ISPs was checked by the Ion Sphere™  
209 Quality Control assay (Life Technologies, Paisley, UK) on the Qubit® 2.0  
210 Fluorometer (Life Technologies) and then enriched by the Ion OneTouch™ ES  
211 Instrument before loading onto a 316™ chip. Sequencing was carried out using the  
212 Ion PGM™ Sequencing 200 Kit v2 (Life Technologies) and the Ion Torrent PGM™.

### 213 **Bioinformatics for *RHD* LR-PCR**

214 Torrent Suite™ Software Version 4.4 was utilised in order to generate a summary  
215 sequencing report indicating the number of reads generated by the sequencer, the  
216 percentage of chip loading and the sequencing files. The FastQC software was run  
217 to assess the quality control across the reads generated (17). The sequencing  
218 samples were aligned to the human genome reference sequence (hg19) using the  
219 Binary Alignment/Map (BAM) and were visualised using Integrative Genome Viewer  
220 (IGV) Version 2.3.46.

221 The samples were annotated using the Variant Call Format (VCF) files to obtain the  
222 SNPs and indels to analyse the genotype and predict the phenotype. Antigens were  
223 determined by choosing the right transcript according to the Blood Group Antigen

224 Factsbook (18). Each antigen was determined by its chromosomal location, the type  
225 of variant (SNP or indel), gene, the reference nucleotide, the changing nucleotide,  
226 depth of coverage, the transcript used in analysis based on the NCBI database, the  
227 location of the variant (intronic or exonic), codon, an exon number of that variant, an  
228 amino acid substitution and the position of the nucleotide change. The SeattleSeq  
229 Annotation tool 141 site was used to annotate the sequencing data of the LR-PCR  
230 approach (19). By using Browser Extensible Data (BED) files, the bedtools website  
231 was used to mask the *RHCE* gene in order to analyse the *RHD* gene (20). The  
232 *RHCE* gene was annotated by 'Ns' on its sequencing nucleotides.

### 233 ***RHD* Intronic SNP sequencing**

234 gDNA samples from blood donors of different phenotypes were tested. *RHD*-specific  
235 primers amplified the regions around the intronic SNPs (Table 2). Two different  
236 enzymes were used, BioMix™ 2X master mix (Bioline Reagents Limited, United  
237 Kingdom) or Q5® Hot Start High-Fidelity 2X Master Mix, (New England Biolabs,  
238 United Kingdom). A 50 µL PCR reaction was prepared containing 1X master mix,  
239 200ng of DNA template, 1 µM of each of the primers. Cycling was carried out on a  
240 Veriti Thermal Cycler (Life Technologies) following optimised conditions; 95°C for 10  
241 min, 35 cycles of 95°C for 30 s and optimised annealing temperature for 1 min, 72°C  
242 for 30 s, followed by a final 72°C step for 10 min. To validate PCR amplification, PCR  
243 products were run on a 1% w/v agarose gel in 1x TAE buffer. PCR products were  
244 purified using the QIAquick Gel Extraction Kit, (Qiagen Ltd, West Sussex, United  
245 Kingdom) according to the manufacturer's instructions. PCR amplicons were  
246 subjected to Sanger sequencing by Eurofins Genomics. Results were aligned with  
247 the human genome reference sequence (hg19). CodonCode Aligner 6.0 software  
248 was used to analyse the data.

## 249 Results

### 250 Determination of *RHD* Zygosity

251 For zygosity testing, the presence or absence of *RHD* amplification on the ddPCR  
252 platform was used to determine whether the samples were *RHD* negative or *RHD*  
253 positive, respectively. The mean number of copies per droplet for all molecules was  
254 0.15 (0.03 – 0.57) for plasma DNA samples and 0.39 (0.05 – 0.69) for buffy coat  
255 DNA samples. The ratio of *RHD5* (FAM)/ *AGO1* (HEX) and *RHD7* (FAM)/ *AGO1*  
256 (HEX) generated by the QuantaSoft v1.2 Software was then used to determine  
257 whether the D-positive samples were hemizygous or homozygous for the *RHD* gene.  
258 Samples showing ratios close to 1 were determined to be homozygous *RHD* positive  
259 and samples with ratios closer to 0.5 were classified as hemizygous *RHD* positive  
260 (Figure 2).

261 The results demonstrated that the assay worked equally well on cell free DNA and  
262 gDNA for zygosity determination (Table 3) (Figure 2). Three rr control samples were  
263 tested (147J, 1660, 7807) and results demonstrated amplification of only the  
264 reference (*AGO1*), giving a ratio of zero (Figure 2). The hemizygous D+ R<sub>0</sub>r  
265 (Dce/dce) (*n* = 8), R<sub>1</sub>r (DCe/dce) (*n* = 12) and R<sub>2</sub>r (DcE/dce) (*n* = 1) samples  
266 demonstrated ratios close to 0.5 as expected (Table 3) (Figure 2), except for sample  
267 1777. Sample 1777, previously classified by serology as being phenotypically R<sub>1</sub>r  
268 (DCe/dce), expressed ratios of 0.97 and 1.04 for the *RHD5* and *RHD7* multiplex  
269 reactions, respectively (Table 3). This result contradicted previous serological  
270 classification and indicated that the sample expressed two copies of the *RHD* gene.  
271 Therefore, it is more feasible that this sample actual expresses the R<sub>1</sub>R<sub>0</sub> (DCe/DCe)  
272 phenotype. The homozygous D+ R<sub>1</sub>R<sub>1</sub> (DCe/DCe) (*n* = 13), R<sub>2</sub>R<sub>2</sub> (DcE/DcE) (*n* = 5),

273  $R_1R_2$  (DCE/dCE) ( $n = 10$ ) and  $R_2R_2$  (DCE/DCE) ( $n = 1$ ) samples were expected to  
274 generate a ratio close to 1, and this was achieved in 90% of samples. Sample 087W  
275 was serologically typed as expressing the  $R_2R_2$  (DCE/dCE) phenotype. However, the  
276 dPCR results demonstrate that this sample is hemizygous for the *RHD* gene, since  
277 both assays illustrated a ratio close to 0.5 (Figure 2). Therefore, it is likely that  
278 sample 087W has the  $R_2r''$  (DCE/dce) genotype as opposed to the  $R_2R_2$  (DCE/DCE)  
279 serologically predicted genotype. Further sequencing analysis was required to  
280 determine the actual genotype of the incorrectly labelled  $R_1R_2$  samples (729M and  
281 351D) (Figure 2).

## 282 ***RHD* intronic polymorphisms**

283 We sequenced the complete *RHD* gene from individuals with defined *RH* genotypes  
284 using LR-PCR (Table 2) and we identified several intronic polymorphisms that  
285 closely correlated with the individuals *DCE* status. On further analysis using Sanger  
286 sequencing, five SNPs showed complete concordance when scrutinised using  
287 primers flanking these regions (Tables 2 and 4).

## 288 **Comparison of *RHD* intronic polymorphisms and zygosity**

289 Two of the  $R_1R_2$  (DCE/dCE) presumed genotype samples tested (729M and 351D)  
290 expressed ratios close to 0.5 for both assays (Figure 2b). Since sample 729M has  
291 also been typed as weak D, it is highly unlikely that this sample is homozygous *RHD*  
292 positive. Therefore, it is clear this sample has been misclassified as  $R_1R_2$ , but we  
293 could not ascertain whether the true genotype for sample 729M was  $R_2r'$  (DCE/dCE),  
294  $R_2r$  (DCE/dce),  $R_0r^y$  (Dce/dCE) or  $R_1r''$  (DCE/dce). Consequently, LR-PCR coupled  
295 with NGS revealed that sample 729M displayed the exon 9 Gly385Ala 1154G>C  
296 SNP, and thus was classified as weak D type 2. In addition, the sample illustrated

multiple *RHD* intronic SNPs which appear to be associated with the R<sub>2</sub> (DcE) haplotype, which demonstrates that sample 729M is likely to be R<sub>2</sub>r' (DcE/dCe) (Table 4). Sample 351D was not typed serologically as weak D but the dPCR data shows that only one copy of *RHD* is present (Figure 2b) and thus the genotype must either be R<sub>2</sub>r' (DcE/dCe), R<sub>Z</sub>r (DCE/dce), R<sub>0</sub>r<sup>y</sup> (Dce/dCE) or R<sub>1</sub>r'' (DCe/dcE). This sample did not show the R<sub>2</sub> associated *RHD* intronic SNPs and hence is likely to have a genotype of R<sub>1</sub>r'' (DCe/dcE), R<sub>0</sub>r<sup>y</sup> (Dce/dCE) or R<sub>Z</sub>r (DCE/dce).

## Discussion

*RHD* zygosity assignment has proved to be a useful diagnostic tool in the clinical management of HDFN. Here, determination of homozygous (*RHD/RHD*) fathers would give confidence (assuming paternity) of prenatal prediction of D-positive fetuses, and signal where further monitoring or administration of prophylactic anti-D maybe required. Without doubt, the most appropriate technique would be the assessment of D-positive infants directly by analysis of free fetal DNA in maternal plasma. However, in repeat pregnancies fathered by *RHD/RHD* homozygotes maternal plasma testing would not be necessary as the fetus would invariably be D-positive. This is of course with the caveat that paternity can be assured during the maternal consenting process. Previous methods have utilised qPCR (4, 7-9), MLPA (6), mass spectrometry (10) and analysis of the *Rhesus box* (2, 5, 11). However, as we have previously mentioned, individuals have been described that confound zygosity testing when relying on analysis of the *Rhesus box* repeat sequences (11). Here we describe a rapid and accurate further method for defining *RHD* zygosity. We have used this on a small cohort of phenotyped blood samples and demonstrated that this method could be used effectively to define paternal zygosity,

321 and in addition, to correct presumed phenotype in blood donors which is presently  
322 dependent on phenotype prediction.

323 In three samples we have analysed, and a weak D sample, we have clearly  
324 demonstrated homo and hemizyosity for *RHD*, which was not in concordance with  
325 predicted phenotype. The vast majority of current genotyping methods (22-27) are  
326 not able to define zygoty (except the study by Gassner *et al.* (10) or unless an  
327 assessment of intronic *RHD*-specific SNPs is performed, some of which are  
328 described in this paper). Our description of candidate SNPs that define the *RHD*  
329 gene within the DcE haplotype will also provide a method to differentiate homo or  
330 hemizyosity, and we have candidate *RHD* intronic SNPs that define the DcE and  
331 Dce *RHD* genes (in preparation). However, much more work on a larger number of  
332 donors (including the testing of rare RH haplotypes) has to be done before these  
333 candidate *RHD* intronic SNPs can be confirmed as being truly DcE and Dce specific.  
334 Nevertheless, these *RHD* intronic SNPs may not be able to differentiate between  
335 DcE/DcE and DcE/dce; DcE/DcE and DcE/dce; and Dce/Dce and Dce/dce  
336 genotypes, however, the dPCR method described here is able to facilitate this  
337 (differentiating homo and hemizyosity). Clearly, for these candidate SNPs to have  
338 clinical utility, a larger cohort of phenotyped samples will require sequencing. We  
339 have subsequently performed such an analysis on 37 Rh phenotyped individuals,  
340 and have found complete concordance with the five DcE-associated candidate SNPs  
341 described in this study. We have identified a further 11 such candidate SNPs that  
342 also are in concordance with DcE genotype. (WAT, KAS, AJH, MK, TEM and NDA,  
343 manuscript in preparation). We are currently investigating a number of Rh variants  
344 and rare phenotypes (e.g. Rz) to assist in their identification.



This method provides a quick and accurate platform for rapid determination of *RHD* zygosity. In this small cohort of samples, we would be unlikely to see rare haplotypes such as DCE, dCe, dcE and Dce. However, both dCe and dcE haplotypes were identified. Further zygosity-based studies are clearly necessary to reassess the population frequencies of these D-negative haplotypes. It is important also to consider that fathers that are *RHD* hemizygous DCE/dCe or DcE/dcE may pass the dCe or dcE haplotypes to their children, and these fetuses may be at risk of HDFN due to anti- C or G (28) or anti-E (29). Fetal genotyping for inheritance of both Rh C and Rh E has been routinely performed using maternal plasma and should therefore be used in such cases where hemizygosity has been defined. We believe that the method we describe here is a useful addition to the diagnostic repertoire available to the clinician in the management of HDFN.

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**Table 1:** *RHD5*, *RHD7* and *AGO1* oligonucleotide sequences, product size and gene location.

Amplicon location	Multiplex Reaction	Primer	Sequence (5' - 3')	Dual-Labelled Hydrolysis Probe (5' - 3')	Length (bp)
1p36.11 <i>RHD</i> Exon 5	1	<i>RHD5</i> Forward*	CGCCCTCTTCTTG TGGATG	FAM- TCTGGCCAAGTTTCA ACTCTGCTCTGCT-BHQ1	82bp
		<i>RHD5</i> Reverse*	GAACACGGCATTCTTCCTTTC		
1p36.11 <i>RHD</i> Exon 7	2	<i>RHD7</i> Forward*	CAGCTCCATCATG GGCTACAA	FAM- AGCTTGCTGGGTCTG CTTGGAGAGATC-BHQ1	75bp
		<i>RHD7</i> Reverse*	AGCACCAGCAGCA CAATGTAGA		
1p34.3	1 and 2	<i>AGO1</i> Forward**	GTTCCGGCTTTTCA CAGTCT	HEX- CTGCCATGTGGAAGATG ATG -BHQ1	81bp
		<i>AGO1</i> Reverse**	CTCCATAGCTCTC CCCACTC		

\*Taken from Finning *et al.* (15)

\*\*Taken from Fan, *et al.* (14)

**Table 2:** *RHD* intronic SNP and *RHD* long-range PCR oligonucleotide sequences, product sizes and corresponding SNP in the *RHD* gene (hg19 human genome reference sequence, for intronic SNPs).

Intronic SNPs	Intron	Forward Primer Sequence 5'-3'	Reverse Primer Sequence 5'-3'	Length (bp)
25,611,580 G>A	2	TTTTACTGGACAGCCCTACT CC	CATGGCTATTTATTGTCTA GCAGCA	558
25,614,400 C>G	2	GCTACCATGCCCTGCTAAT	TCCAGTACTTTTCAGAGC C	417
25,625,471 T>C	3	GGGGCAGCTTCATCTTATC AAGAG	CTCACTGCAACCTCCACC CGTT	419
25,627,066 C>G	3	TGGGATTACAGGCAAAATT AG	AGGTGTGACTTGAAGCCA T	834
25,648,349 T>C	8	TCCAGGAATGACAGGGCT	TGAGGACTGCAGATAGGG	525
<b><i>RHD</i> exons covered</b>				
1-3	1,2	GATTGGGTCCGTGATTGGC ATT	GGCCGCGGGAATTCGATT GTTGTCTTTATTTTCAAA ACCCT	22,829
2-7	2-6	GCCGCGAATTCAGTAGTGT GACGAGTGAACTCTATCT CGAT (Ds2-s*)	GGCCGCGGGAATTCGATT GAGGCTGAGAAAGGTTAA GCCA	23,610
7-10	7-9	GCCGCGAATTCAGTAGTGA CAAAC TCCCCGATGATGTG AGTG	GGCCGCGGGAATTCGATT GTGGTACATGGCTGTATT TTATTG	22,731

\*Adapted from Legler *et al.* (16)



458 **Table 3:** Zygosity testing results determined by ratio analysis for DNA samples  
 459 extracted from both the plasma (cfDNA) and buffy coat (gDNA) of human whole  
 460 blood samples.

Sample	<i>RH</i> <sup>1</sup>	Ratio ( <i>RHD5</i> (FAM) / <i>AGO1</i> (HEX))	Ratio ( <i>RHD7</i> (FAM) / <i>AGO1</i> (HEX))	Hemizygous or homozygous	Genotype determined by dPCR**
147J*	rr (dce/ dce)	0	0	Homozygous <i>RHD</i> negative	rr (dce/ dce)
1660*		0	0	Homozygous <i>RHD</i> negative	rr (dce/ dce)
7807*		0	0	Homozygous <i>RHD</i> negative	rr (dce/ dce)
9763*	<i>R</i> <sub>0</sub> r (Dce/ dce)	0.45	0.43	Hemizygous	<i>R</i> <sub>0</sub> r (Dce/ dce)
069F*		0.5	0.49	Hemizygous	<i>R</i> <sub>0</sub> r (Dce/ dce)
740B*		0.47	0.46	Hemizygous	<i>R</i> <sub>0</sub> r (Dce/ dce)
258D*		0.51	0.51	Hemizygous	<i>R</i> <sub>0</sub> r (Dce/ dce)
(079*)*		0.51	0.50	Hemizygous	<i>R</i> <sub>0</sub> r (Dce/ dce)
649B*		0.5	0.5	Hemizygous	<i>R</i> <sub>0</sub> r (Dce/ dce)
8931*		0.49	0.49	Hemizygous	<i>R</i> <sub>0</sub> r (Dce/ dce)
5784*		0.49	0.50	Hemizygous	<i>R</i> <sub>0</sub> r (Dce/ dce)
065S*		0.49	0.49	Hemizygous	<i>R</i> <sub>1</sub> r (DCE/ dce)
118Z*	<i>R</i> <sub>1</sub> r (DCE/ dce)	0.5	0.49	Hemizygous	<i>R</i> <sub>1</sub> r (DCE/ dce)
1226*		0.52	0.51	Hemizygous	<i>R</i> <sub>1</sub> r (DCE/ dce)
1306*		0.51	0.53	Hemizygous	<i>R</i> <sub>1</sub> r (DCE/ dce)
1777*		0.97	1.04	Homozygous <i>RHD</i> positive	<i>R</i> <sub>1</sub> <i>R</i> <sub>0</sub> (DCE/ Dce)
180H*		0.52	0.52	Hemizygous	<i>R</i> <sub>1</sub> r (DCE/ dce)
181F*		0.52	0.49	Hemizygous	<i>R</i> <sub>1</sub> r (DCE/ dce)
148R <sup>2</sup>		0.50	0.50	Hemizygous	<i>R</i> <sub>1</sub> r (DCE/ dce)
6418 <sup>2</sup>		0.51	0.49	Hemizygous	<i>R</i> <sub>1</sub> r (DCE/ dce)
3093		0.51	0.51	Hemizygous	<i>R</i> <sub>1</sub> r (DCE/ dce)
572R <sup>2</sup>		0.50	0.50	Hemizygous	<i>R</i> <sub>1</sub> r (DCE/ dce)
7687 <sup>2</sup>		0.50	0.51	Hemizygous	<i>R</i> <sub>1</sub> r (DCE/ dce)
5481 <sup>2</sup>	<i>R</i> <sub>2</sub> r (DcE/ dce)	0.50	0.51	Hemizygous	<i>R</i> <sub>2</sub> r (DcE/ dce)
1220*	<i>R</i> <sub>1</sub> <i>R</i> <sub>1</sub> (DCE/ DCE)	0.98	1.01	Homozygous <i>RHD</i> positive	<i>R</i> <sub>1</sub> <i>R</i> <sub>1</sub> (DCE/ DCE)
131Z*		0.99	1.04	Homozygous <i>RHD</i> positive	<i>R</i> <sub>1</sub> <i>R</i> <sub>1</sub> (DCE/ DCE)
165F*		0.94	0.9	Homozygous <i>RHD</i> positive	<i>R</i> <sub>1</sub> <i>R</i> <sub>1</sub> (DCE/ DCE)
1793*		0.99	1	Homozygous <i>RHD</i> positive	<i>R</i> <sub>1</sub> <i>R</i> <sub>1</sub> (DCE/ DCE)
0670*		0.91	0.85	Homozygous <i>RHD</i> positive	<i>R</i> <sub>1</sub> <i>R</i> <sub>1</sub> (DCE/ DCE)
1347*		0.99	1.03	Homozygous <i>RHD</i> positive	<i>R</i> <sub>1</sub> <i>R</i> <sub>1</sub> (DCE/ DCE)
138R*		0.95	0.98	Homozygous <i>RHD</i> positive	<i>R</i> <sub>1</sub> <i>R</i> <sub>1</sub> (DCE/ DCE)
052M		0.99	1.03	Homozygous <i>RHD</i> positive	<i>R</i> <sub>1</sub> <i>R</i> <sub>1</sub> (DCE/ DCE)
247X		1.02	1.01	Homozygous <i>RHD</i> positive	<i>R</i> <sub>1</sub> <i>R</i> <sub>1</sub> (DCE/ DCE)
078U		0.99	1.01	Homozygous <i>RHD</i> positive	<i>R</i> <sub>1</sub> <i>R</i> <sub>1</sub> (DCE/ DCE)
103N		1.01	1.03	Homozygous <i>RHD</i> positive	<i>R</i> <sub>1</sub> <i>R</i> <sub>1</sub> (DCE/ DCE)
1461		0.99	1.01	Homozygous <i>RHD</i> positive	<i>R</i> <sub>1</sub> <i>R</i> <sub>1</sub> (DCE/ DCE)
877L		1.01	0.98	Homozygous <i>RHD</i> positive	<i>R</i> <sub>1</sub> <i>R</i> <sub>1</sub> (DCE/ DCE)
658G	<i>R</i> <sub>2</sub> <i>R</i> <sub>2</sub> (DcE/ DcE)	1.02	1.03	Homozygous <i>RHD</i> positive	<i>R</i> <sub>2</sub> <i>R</i> <sub>2</sub> (DcE/ DcE)
738W		1.02	1.04	Homozygous <i>RHD</i> positive	<i>R</i> <sub>2</sub> <i>R</i> <sub>2</sub> (DcE/ DcE)
087W		0.51	0.49	Hemizygous	<i>R</i> <sub>2</sub> r <sup>''</sup> (DcE/ dcE)

132H		1.01	1.03	Homozygous <i>RHD</i> positive	R <sub>2</sub> R <sub>2</sub> (DcE/ DcE)
689U		0.99	1.01	Homozygous <i>RHD</i> positive	R <sub>2</sub> R <sub>2</sub> (DcE/ DcE)
729M <sup>2</sup>		0.50	0.49	Hemizygous	R <sub>1</sub> r' (DcE/ dcE) or R <sub>2</sub> r' (DcE/ dcE)
896H		0.98	1.03	Homozygous <i>RHD</i> positive	R <sub>1</sub> R <sub>2</sub> (DcE/ DcE)
898D		0.99	0.97	Homozygous <i>RHD</i> positive	R <sub>1</sub> R <sub>2</sub> (DcE/ DcE)
351D		0.51	0.51	Hemizygous	R <sub>1</sub> r' (DcE/ dcE) or R <sub>2</sub> r' (DcE/ dcE)
9316	R <sub>1</sub> R <sub>2</sub> (DcE/ DcE)	1.02	1.01	Homozygous <i>RHD</i> positive	R <sub>1</sub> R <sub>2</sub> (DcE/ DcE)
911E		1.02	1.03	Homozygous <i>RHD</i> positive	R <sub>1</sub> R <sub>2</sub> (DcE/ DcE)
4195		1.02	1.01	Homozygous <i>RHD</i> positive	R <sub>1</sub> R <sub>2</sub> (DcE/ DcE)
645C		1.06	1.03	Homozygous <i>RHD</i> positive	R <sub>1</sub> R <sub>2</sub> (DcE/ DcE)
3627		0.99	1.01	Homozygous <i>RHD</i> positive	R <sub>1</sub> R <sub>2</sub> (DcE/ DcE)
8873		1.02	1.03	Homozygous <i>RHD</i> positive	R <sub>1</sub> R <sub>2</sub> (DcE/ DcE)
746P		1.02	0.99	Homozygous <i>RHD</i> positive	R <sub>1</sub> R <sub>2</sub> (DcE/ DcE)
	R <sub>2</sub> R <sub>2</sub> (DcE/ DcE)				

<sup>1</sup> Serologically predicted phenotype provided by National Health Service Blood and Transplant (NHS BT) (Bristol, UK).

<sup>2</sup> Sample is Weak D.

\*DNA samples tested from plasma.

\*\* The C/c and E/e status based on serological information. Only the D/d genotype was corrected by dPCR.

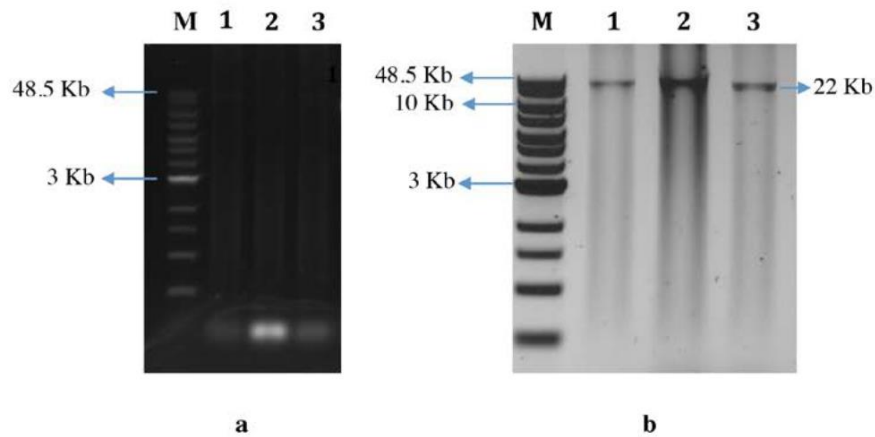
**Table 4:** *RHD* intronic SNP sequencing and *RHD* LR-PCR NGS results for a range of DNA samples.

Intronic SNPs in <i>RHD</i> (R <sub>1</sub> /R <sub>1</sub> to R <sub>2</sub> )	<i>RHD</i> Intron	R <sub>1</sub> R <sub>1</sub> (DcE/ DcE) (n=4)	R <sub>1</sub> r (DcE/ dce) (n=1)	R <sub>0</sub> r (DcE/ dce) (n=8)	R <sub>2</sub> R <sub>2</sub> (DcE/ DcE) (n=6)	R <sub>2</sub> r (DcE/ dce) (n=1)	R <sub>1</sub> R <sub>2</sub> (DcE/ DcE) (n=5)	Sample 729M (n=1)	Sample 351D (n=1)
25,611,580 G>A	2	G/G	G	G	A/A	A	G/A	A	G
25,614,400 C>G rs28718098*	2	C/C	C	C	G/G	G	C/G	G	C
25,625,471 T>C rs2904843*	3	T/T	T	T	C/C	C	T/C	C	T
25,627,066 C>G rs2986167*	3	C/C	C	C	G/G	G	C/G	G	C
25,648,349 T>C rs28669938*	8	T/T	T	T	C/C	C	T/C	C	T

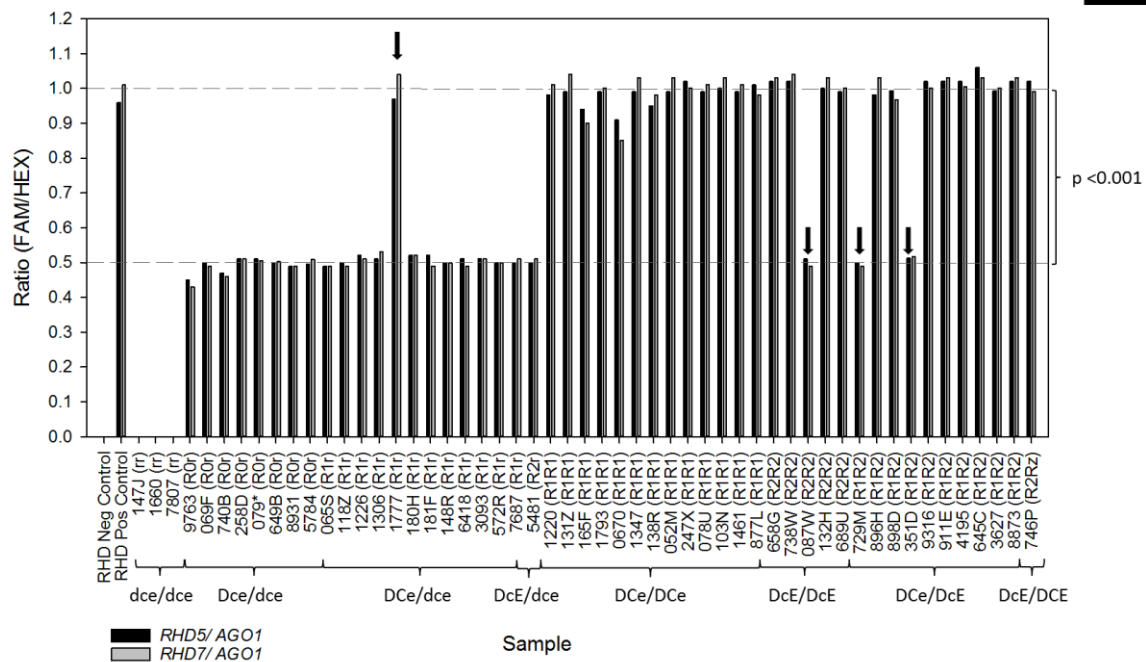
\* Taken from the National Center for Biotechnology Information (NCBI) (16).

The table indicates the serologically inferred genotype of the samples provided by the National Health Service Blood and Transplant (NHS BT) (Bristol, UK).

**Figure 1:** LR-PCR products for the Rh blood group system. Three long-range amplicons (1, 2, and 3) were designed to amplify the entire *RHD* gene. (a) An RhD-negative sample shows no bands for the *RHD* LR-PCR in lanes 1, 2 and 3, which represent the three amplicons. (b) An RhD-positive sample gives amplification of all three products, with each product being about 22 kb.



**Figure 2:** Ratio analysis to determine zygosity using two multiplex reactions (*RHD5* (FAM)/*AGO1* (HEX) and *RHD7* (FAM)/*AGO1* (HEX) for samples with varying Rh phenotypes. The grey dotted lines at 0.5 and 1 on the y axis represent the ratio generated by hemizygous D+ samples and homozygous D+ samples, respectively. The mean ratio for hemizygous and homozygous D+ positive samples for both plasma and buffy coat extracted samples (Table 3) illustrated significant difference ( $p < 0.001$ ). The arrows indicate the samples that illustrated discordant results compared with the serologically predicted genotype.



492 **Supplemental Figure 1:** Annealing temperature gradient (60°C, 58.4°C, 57.4°C and  
493 56°C) of dPCR for both multiplex reactions (*RHD5* (FAM)/*AGO1* (HEX) and *RHD7*  
494 (FAM)/*AGO1* (HEX)) using sample 0745 (homozygous for *RHD*) extracted from  
495 human whole blood and a NTC. a) Optimisation of the *RHD5* (FAM) and *AGO1*  
496 (HEX) multiplex reaction. The results illustrate that separation is visible for both  
497 targets at all annealing temperatures. Marginally greater separation is visible at  
498 56°C, but all annealing temperatures are no more than 0.03-0.04 away from a ratio  
499 of 1. b) Optimisation of the *RHD7* (FAM) and *AGO1* (HEX) multiplex reaction. The  
500 results illustrate that separation is visible for both targets at all annealing  
501 temperatures. However, optimal separation was determined to be 58.4°C, since this  
502 temperature expressed a ratio closer to 1 (0.995).

